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(54) Title: ANALYTICAL DEVICE

(57) Abstract

A biosensor comprises a layer of dielectric material, at least a part of which is coupled to a biocompatible porous matrix containing immobilised biochemicals. The biosensor may be based on the principle of frustrated total reflection (FTR). Most conveniently, the porous matrix is a hydrogel, e.g. a hydrogel selected from the group consisting of polysaccharides, e.g. agarose, dextran, carrageenan, alginic acid, starch, cellulose, and derivatives thereof, e.g. carboxymethyl derivatives, xanthan gum, pectin, and a water-swellable organic polymer such as polyvinyl alcohol, polyacrylic acid, polyacrylamide, and polyethylene glycol.

LEDIGLICH ZUR INFORMATION

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Title : Analytical Device

This invention relates to sensors, in particular to chemical or biochemical sensors having a dielectric surface layer, such as those based on the principle of frustrated total internal reflection.

Many devices for the automatic determination of biochemical analytes in solution have been proposed in recent years. Typically, such devices (biosensors) include a sensitised coating layer which is located in the evanescent region of a resonant field. Detection of the analyte typically utilizes optical techniques such as, for example, surface plasmon resonance (SPR), and is based on changes in the thickness and/or refractive index of the coating layer resulting from interaction of that layer with the analyte. This causes a change, eg in the angular position of the resonance.

One of the techniques which has been suggested for use in optical biosensors is frustrated total reflection. The principles of frustrated total reflection (FTR) are well-known; the technique is described, for example, by Bosacchi and Oehrle [Applied Optics (1982), 21, 2167-2173]. An FTR device for use in immunoassay is disclosed in US Patent 4,857,273 and comprises a cavity layer bounded on one side by the sample under investigation and on the other side by a spacer layer which in turn is mounted on a substrate. The substrate-spacer layer interface is irradiated with monochromatic radiation such that total reflection occurs, the associated evanescent field penetrating through the spacer layer. If the thickness of the spacer layer is correct and the incident parallel wave vector matches one of the resonant mode propagation constants, the total reflection is frustrated and radiation is coupled into the cavity layer. The cavity layer must be composed of material which has a higher refractive index than the spacer layer and which is transparent at the wavelength of the incident radiation.

More recently, FTR biosensors have been described [see, for example, PCT Patent Application WO 90/06503] in which the cavity layer is a thin film of relatively high refractive index material, typically an inorganic oxide.

In all biosensors, it is necessary that the sensitised coating layer comprise a layer of immobilised chemical or biochemical species. It has recently been suggested, in connection with SPR sensors, that a biocompatible porous matrix such as a hydrogel be employed to achieve this immobilisation [see PCT Patent Application WO 90/05303]. The matrix typically comprises a dextran which is coupled to the sensor surface (which in SPR is a metal layer) via a linking group, preferably a thiol or a disulphide. Thiol or disulphide linking groups are, however, unsuitable for directly linking a porous matrix to the oxide surface of an FTR-based sensor.

We have now found, however, that a biocompatible porous matrix may be used to immobilise the sensitive coating on the surface of a biosensor with a dielectric surface layer, and that this approach offers unexpected advantages over previously-known sensors.

According to the invention, there is provided a biosensor comprising a layer of dielectric material, at least a part of which is coupled to a biocompatible porous matrix containing immobilised biochemicals.

The sensor according to the invention is advantageous in that a much higher density of immobilised biochemicals can be obtained compared with immobilisation of the biochemical species directly on the sensor surface. This results in an enhanced measuring signal and a greater dynamic range. Also, binding activity of the immobilised species is increased and undesirable desorption of the immobilised species, eg caused by treatment with detergents, is reduced. The porous matrix provides a three-dimensional matrix for binding of analyte

molecules in a sample under investigation, increasing the effect of such binding on the refractive index of the surface region.

The porous matrix also renders the sensor surface chemically resistant to the sample media with which it is brought into contact during use, and is compatible with proteins and other biomolecules. A great variety of biomolecules may be immobilised in the porous matrix by covalent binding, which enhances the versatility of the sensor, in terms of the range of analytes which may be detected.

In addition, greater sensitivity may be achieved than is possible using the SPR technique, even where the porous matrix is employed as described in WO 90/05303. The enhanced sensitivity may be due, for example, to the fact that the propagation distance of the light coupled into the cavity layer may be optimised, eg by appropriate choice of materials and layer thicknesses. In addition, it may be possible, by appropriate choice of device parameters, to confine the evanescent field more closely to the surface of the dielectric layer than is possible with corresponding SPR devices (the choice of parameters being made according to the principle described in European Patent No 0075353).

Coupling of the porous matrix may also be achieved more easily on the inorganic oxide surface of the sensor according to the invention than on the metal surface of an SPR sensor. Also, the fabrication of the dielectric structure of the sensor of the present invention may be simpler and less costly than that of SPR-based sensors involving thin layers of metal. In addition, the inorganic oxide surface of the sensor is not susceptible to tarnishing, as are silver or gold surfaces, or to oxidation, as is the case with aluminium surfaces.

The sensor according to the invention may be based on the principle of FTR, in which case the dielectric layer forms the

cavity layer of the sensor.

The porous matrix may have a thickness in the range of from a few nm, eg about 5nm, to several hundred nm.

The porous matrix is most preferably a hydrogel, eg one of those described by Merrill et al [Hydrogels in Medicine and Pharmacy, Vol III, (1986), Ed. Pappas NA, Chapter 1, CRC Press].

Examples of suitable hydrogels include polysaccharides, eg agarose, dextran, carrageenan, alginic acid, starch, cellulose, or derivatives thereof, eg carboxymethyl derivatives, xanthan gum, pectin, or a water-swellable organic polymer such as polyvinyl alcohol, polyacrylic acid, polyacrylamide, and polyethylene glycol.

Due to its well-known applicability as a binding medium for biomolecules in chromatography, dextran is a particularly preferred hydrogel.

In order to facilitate coupling of the porous matrix on the surface of the sensor, the surface of the dielectric layer may be derivatised or activated so as to provide coupling sites for the porous matrix.

For example, the layer may be reacted with a silane-based coupling compound in a known manner. A suitable such reagent is, for example, a terminal amino-alkyl trimethoxysilane, eg the 3-aminopropyl compound, used at a concentration of about 2% w/v in acetone. Details of immobilisation techniques using this reagent have been described by Weetall [see, for example, US Patent 3,652,761 and "Immobilised Biochemicals and Affinity Chromatography", R B Dunlop (Ed), Plenum Press, New York (1974), pp191-212].

After reaction with the amino-silane reagent, the amino

terminals immobilised on the dielectric layer may in turn be reacted with succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), then 2-mercaptoethanol, followed by epichlorhydrin for cross-linking to the porous matrix, excess reagents then being removed and the activated surface with immobilised epoxide groups then being treated with a solution of the species constituting the porous matrix.

An alternative method for coupling the porous matrix to the layer surface involves treatment with epoxy-silane reagents, especially glycidyloxypropyltrimethoxysilane, eg at a concentration of about 2% v/v in toluene for about 2 hours at 70°C as described by Herman et al [J Chromatogr Sci (1981), 19(9), 470-476]. In this method the use of aldehyde reagents is unnecessary, since the epoxysilylated dielectric layer can react directly with the porous matrix molecules.

In a preferred method, however, the porous matrix is coupled directly to the dielectric layer surface ie without derivatisation or activation of the surface.

Dextran in particular may be coupled directly to the dielectric surface and it is found that devices prepared in this way have improved properties. In particular it is found that there may be an increase in the density of immobilised biomolecules. This may be a result of a higher dextran concentration at the surface. Devices with dextran coupled directly to the surface may nonetheless be as stable as devices in which the dextran is coupled to the surface indirectly. In particular, in both cases various treatments, eg washing at 70°C in 6M urea or washing in high concentrations of detergents, do not remove the dextran.

Coupling of the porous matrix to the dielectric surface may involve derivatisation of the porous matrix molecules, eg carboxymethylation. Since such derivatisation may involve treatment with reagents which may have a detrimental effect on

the surface, it is frequently preferred to perform the derivatisation prior to contact with the surface.

Following linkage of the porous matrix with the dielectric layer, biochemicals such as antibodies for a particular analyte are immobilised in the matrix. It may be necessary to first activate the matrix. For example, a hydrazide function may be created in a dextran matrix for binding biomolecules containing aldehyde groups, eg antibodies in which the carbohydrate chain has been oxidised. The dextran matrix is initially modified with carboxymethyl groups which are partly reacted to form hydrazide groups.

Alternatively, reactive ester functions may be formed by modification of some of the carboxyl groups in carboxymethyl-modified dextran, eg by treatment with an aqueous solution of N-hydroxysuccinimide and N-(3-dimethyl-aminopropyl)-N'-ethylcarbodiimide hydrochloride. Biomolecules such as proteins, peptides and amino-modified oligonucleotides which contain amino groups may be coupled to the matrix via activated carboxyl groups.

In another method, the reactive ester function is reacted with a disulphide-containing species such as 2-(2-pyridinyldithio)ethanamine. The disulphide containing matrix so obtained can be used to couple thiol-containing biomolecules such as reduced F(ab) fragments of immunoglobulins. Alternatively, disulfide-containing ligands, eg N-succinimidyl 3-(2-pyridinyldithio)propionate (SPDP) modified proteins, may be immobilised after cleavage of the matrix disulphide bonds, eg by reduction or thiol-disulphide exchange.

As an alternative to covalent immobilisation of biomolecules in the porous matrix, immobilisation may also occur by complex formation. For example, the biomolecule may be an antibody to dextran which is conjugated with a specific binding partner

for the analyte.

The hydrogel may incorporate absorbing species, eg dye molecules, which may be linked to the hydrogel molecules. Changes occuring when a sample is contacted with the biosensor surface may be detected as a reduction in the intensity of the reflected radiation, radiation coupled into the dielectric layer being absorbed by the absorbing molecules. This provides a further advantage for the system of the present invention over SPR based sensors.

Typically, an FTR-based biosensor according to the invention will comprise

a) a cavity layer of dielectric material of refractive index n_3,

b) a dielectric substrate of refractive index n_1, and

c) interposed between the cavity layer and the substrate, a dielectric spacer layer of refractive index n_2.

In use, the interface between the substrate and the spacer layer is irradiated with light such that total reflection occurs. In this context, 'light' may include not only visible light but also wavelengths above and below this range, eg in the ultra-violet and infra-red.

Resonant propagation of a guided mode in the cavity layer will occur, for a given wavelength, at a particular angle of incidence of the exciting radiation. Thus, two basic measurement approaches are possible: scanning the angle of incidence at fixed wavelength or scanning the wavelength at a fixed angle of incidence. The former approach, using monochromatic radiation, is preferred since it allows the use of a laser source, simplifying the problem of optical collimation, and avoids dispersion effects, thereby simplifying the analysis of the results.

The angular position of the resonant effect depends on various

parameters of the biosensor device, such as the refractive indices and thicknesses of the various layers. In general, it is a pre-requisite that the refractive index n_3 of the cavity layer and the refractive index n_1 of the substrate should both exceed the refractive index n_2 of the spacer layer. Also, since at least one mode must exist in the cavity to achieve resonance, the cavity layer must exceed a certain minimum thickness.

The cavity layer is preferably a thin-film of dielectric material. Suitably transmissive dielectric materials for the cavity layer include zirconium dioxide, titanium dioxide, aluminium oxide and tantalum oxide.

The cavity layer may be prepared by known techniques, eg vacuum evaporation, sputtering, chemical vapour deposition or in-diffusion.

The dielectric spacer layer must also be suitably transmissive to the incident radiation and must have a lower refractive index than both the cavity layer and the substrate. The layer may, for example, comprise an evaporated or sputtered layer of magnesium fluoride. In this case an infra-red light injection laser may be used as light source. The light from such a source typically has a wavelength around 800nm. Other suitable materials include lithium fluoride and silicon dioxide. Apart from the evaporation and sputtering techniques mentioned above, the spacer layer may be deposited on the substrate by a sol-gel process, or be formed by chemical reaction with the substrate.

The refractive index of the substrate (n_1) must be greater than that (n_2) of the spacer layer but the thickness of the substrate is generally not critical to the performance of the invention.

By contrast, the thickness of the cavity layer must be so

chosen that resonance occurs within an appropriate range of coupling angles. The spacer layer will typically have a thickness of the order of several hundred nanometres, say from about 200nm to 2000nm, more preferably 500 to 1500nm, eg 1000nm. The cavity layer typically has a thickness of a few tens of nanometres, say 10 to 200nm, more preferably 30 to 150nm, eg 100nm.

It is particularly preferred that the cavity layer has a thickness of 30 to 150nm and comprises a material selected from zirconium dioxide, hafnia, silicon nitride, titanium dioxide, tantalum oxide and aluminium oxide, and the spacer layer has a thickness of 500 to 1500nm and comprises a material selected from magnesium fluoride, lithium fluoride and silicon dioxide, the choice of materials being such that the refractive index of the spacer layer is less than that of the cavity layer.

Preferred materials for the cavity layer and the spacer layer are tantalum oxide and silicon dioxide respectively.

Any convenient source of radiation may be used as the source of the incident light but it is preferable to use monochromatic radiation and the most convenient source of such radiation is a laser. The choice of laser will depend inter alia on the materials used for the various layers of which some examples have already been given.

The scanning of angle may be performed either sequentially or simultaneously ie by varying the angle of incidence of a parallel beam of light or by simultaneously irradiating over a range of angles using a fan-shaped beam of light as described (in connection with SPR) in European Patent Application No 0305109A. In the former case, a single-channel detector may be used which is mechanically scanned over a range of angles; in the latter case, in which a range of angles is irradiated simultaneously, it will generally be necessary to

use a multi-channel detector having angular resolution.

At resonance, the incident light is coupled into the cavity layer by FTR, propagates a certain distance along the cavity layer, and couples back out (also by FTR). The propagation distance depends on the various device parameters but is typically of the order of 1 or 2mm.

In general, at resonance, the reflected light will undergo a phase change and it may be the angular position at which this phase change occurs which is detected.

Changes on the surface of the cavity layer, eg binding of antigen to antibody immobilised in the porous matrix, cause changes in the thickness of the layer of immobilised biochemicals and hence shift the angular position of the resonance. In some formats, there may also be a reduction in the intensity of the reflected light, eg if the immobilised species are absorbing at the wavelength of the incident radiation. In this case, this reduction in intensity may be used to monitor the binding processes.

Example

a1) Coupling of dextran to dielectric surface via silanes

i) FTR biosensor devices with a dielectric cavity layer are washed with acetone for 5 minutes and allowed to air-dry.

ii) The surfaces of the devices are silanised with glycidoxypropyltrimethoxy-silane under vacuum at 140°C for 1 hour.

iii) The devices are coated with a solution of dextran (mw 500,000, 14g) in 40ml 0.1M NaOH for 6 hours at room temperature.

iv) The devices are washed with water.

v) The immobilised dextran is carboxymethylated by incubating the devices with bromoacetic acid (1.3g) in 2M NaOH overnight at room temperature.

vi) The devices are washed with water.

a2) Direct coupling of dextran to dielectric surface

i) FTR biosensor devices with a dielectric cavity layer are washed with acetone for 5 minutes and allowed to air-dry.

ii) The devices are coated with a solution of dextran (mw 500,000, 14g) in 40ml 0.1M NaOH for 4 hours at room temperature.

iii) The devices are washed with water.

iv) The immobilised dextran is carboxymethylated by incubating the devices with bromoacetic acid (1.3g) in 10ml 2M NaOH overnight at room temperature.

v) The devices are washed with water.

a3) Alternative method of direct coupling

i) 10g of a 25% w/w solution of dextran (mw 450,000) in water is mixed with 100ml of 1M bromoacetic acid in 2M NaOH and allowed to react overnight.

ii) The pH of the mixture is set to pH 6-8 with 8M acetic acid.

iii) The mixture is dialysed against H_2O and then freeze-dried.

iv) The freeze-dried product is dissolved to 23% w/w in 0.1M HCl (or H_2O).

v) A 10μl drop of the carboxylated dextran solution is applied to the surface of an FTR biosensor device with a dielectric surface layer, spread across the surface, and left overnight to adsorb.

b) Coupling of biomolecules to hydrogel matrix

i) Activation

Carboxymethylated dextran immobilised on the surface by method a1), a2) or a3) is treated with 0.1M N-hydroxysuccinimide (NHS) and 0.4M N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC) for 10 minutes to activate the carboxyl groups of the dextran to reactive N-hydroxysuccinimide esters.

ii) Coupling

The product of step i) is treated with a 20-100μg/ml solution of antibody/protein/peptide/amino-modified oligonucleotide for 10 minutes in 10mM acetate buffer pH 4.0-5.5.

iii) Deactivation

Unreacted NHS-esters remaining on the surface were deactivated by treatment for 10 minutes with 1M ethanolamine hydrochloride adjusted to pH 8.5.

Claims

1. A biosensor comprising a layer of dielectric material, at least a part of which is coupled to a biocompatible porous matrix containing immobilised biochemicals.

2. A biosensor as claimed in Claim 1, wherein the porous matrix is a hydrogel.

3. A biosensor as claimed in Claim 2, wherein the hydrogel is selected from the group consisting of polysaccharides, eg agarose, dextran, carrageenan, alginic acid, starch, cellulose, and derivatives thereof, eg carboxymethyl derivatives, xanthan gum, pectin, and a water-swellable organic polymer such as polyvinyl alcohol, polyacrylic acid, polyacrylamide, and polyethylene glycol.

4. A biosensor as claimed in Claim 3, wherein the hydrogel comprises dextran or a derivative thereof.

5. A biosensor as claimed in any one of the preceding claims, wherein the porous matrix is coupled directly to the dielectric layer surface without derivatisation or activation of the surface.

6. A biosensor as claimed in Claim 5, wherein the porous matrix comprises dextran or a derivative thereof.

7. A biosensor as claimed in any one of the preceding claims, which comprises
a) a cavity layer of dielectric material of refractive index n_3,
b) a dielectric substrate of refractive index n_1, and
c) interposed between the cavity layer and the substrate, a dielectric spacer layer of refractive index n_2.

8. A biosensor as claimed in Claim 7, wherein the cavity

layer is a thin-film of dielectric material.

9. A biosensor as claimed in Claim 8, wherein the cavity layer has a thickness of 30 to 150nm and comprises a material selected from zirconium dioxide, hafnia, silicon nitride, titanium dioxide, tantalum oxide and aluminium oxide, and the spacer layer has a thickness of 500 to 1500nm and comprises a material selected from magnesium fluoride, lithium fluoride and silicon dioxide, the choice of materials being such that the refractive index of the spacer layer is less than that of the cavity layer.

10. A method of manufacturing an FTR biosensor comprising a cavity layer of dielectric material, at least a part of which is coupled to a biocompatible porous matrix containing immobilised biochemicals, which method comprises the steps of

a) derivatising the porous matrix molecules,

b) coupling the porous matrix molecules to the surface of the cavity layer, and

c) immobilising biomolecules in the matrix.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00992

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/543; G01N33/551

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N ; C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 104 491 (BIOSTAR MEDICAL PRODUCTS, INC.) 4 April 1991 See page 8, line 19- page12, line 17; page 36, line 13- page 37, line 26. ---	1-4
Y	WO,A,9 006 503 (ARES-SERONO RESEARCH & DEVELOPMENT LIMITED PARTNERSHIP) 14 June 1990 cited in the application see page 1 - page 8; figures ---	1-3,7-10
Y	EP,A,0 226 470 (UNILEVER PLC.) 24 June 1987 see column 1 - column 4 ---	1-3,7-10
Y	WO,A,9 106 862 (FISONS PLC) 16 May 1991 see the whole document ---	1-4,7-10
Y	WO,A,9 005 303 (PHARMACIA AB) 17 May 1990 cited in the application see page 1 - page 9 ---	1-4,7-10
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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 AUGUST 1992	14. 08. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	HITCHEN C.E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 254 575 (ARES.SERONO RESEARCH & DEVELOPMENT LIMITED PARTNERSHIP) 27 January 1988 See pages 1,2,7.	1,3,5
A	WO,A,8 907 139 (NOVO INDUSTRI A/S) 10 August 1989 see the whole document	1,2,3,5

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200992 SA 59770

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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